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Synthesis and Biological Activities of Copolymer of N-Alaninylmaleimide with Methacrylic Acid

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N-알라닐말레이미드와 메타크릴산의 공중합체 합성과 생물학적 활성

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= Abstract =

새로운 단량체인 N-alaninylmaleimide(AMI)를 maleic anhydride와 β-alanine으로부터 합성하고 그것의 중합체인 poly(AMI)와 poly(AMI-co-MA)를 합성하였다. 얻어진 단량체와 중합체의 구조는 IR과 ^{1}H-NMR spectrophotometer와 원소분석기를 사용하여 확인하였다. GPC를 이용하여 측정된 poly(AMI)와 poly(AMI-co-MA)의 수 평균 분자량은 4,200과 4,000이었다. 합성된 중합체들의 FM-3A와 P-388, 그리고 U-937세포에 대한 세포 독성을 *in vitro*에서, sarcoma 180 세포에 대한 항암 활성을 *in vivo*에서 조사하였다. 그 결과 중합체의 세포독성은 단량체의 세포독성보다 적었으며, 0.8 mg/kg 농도에서 중합체의 항암활성은 다음 순서로 증가됨을 알 수 있었다: 5-fluorouracil < AMI < poly(AMI) < poly(AMI- co-MA).

The new monomer, N-alaninylmaleimide (AMI), was synthesized by the reaction of maleic anhydride and β-alanine. Synthesized AMI, poly(AMI), and poly(AMI-co-MA) were characterized by IR and ^{1}H-NMR spectroscopies, elemental analysis, and gel permeation chromatography (GPC). The number average molecular weights of poly(AMI) and poly(AMI-co-MA) were 4200 and 4000, respectively. *In vitro* cytotoxicities of polymers against mouse mammary carcinoma (FM-3A), mouse leukemia (P-388), and human histiocytic lymphoma (U-937) cell lines were lower than those of AMI. At a dosage of 0.8 mg/kg antitumor activities of samples were increased in the following order: 5-fluorouracil < AMI < poly(AMI) < poly(AMI-co-MA).

Key Words: N-alaninylmaleimide, Poly(N-alaninylmaleimide), Poly(N-alaninylmaleimide-co-methacrylic acid), Average molecular weight, *In vitro* cytotoxicity, *In vivo* antitumor activity

INTRODUCTION

Polymer drugs can be expected to have some advantages such as higher specificity of actions, longer duration of actions and lower toxic side effects compared with low molecular weight drugs. The copolymer of divinyl ether with maleic anhydride (DIVEMA) first reported by Butler has been extensively studied for its broad biological activities such as antitumor, antiviral, antibacterial, interferon-inducing, and antifungal activies.[1,3-5] Many workers has studied in order to obtain a polymeric drug like DIVEMA.[2,6,13,14] We have reported on the studies of syntheses and biological activities of polymeric antitumor agents with the above advantages for polymer drugs.[7-11,15]

The aim of this study is to obtain a new monomer, N-alaninylmaleimide (AMI), and its polymers having biological activity. AMI was expected to show considerably high biological activity because it has amino acid moiety in the repeating unit and its anionic character after hydrolysis is similar to that of DIVEMA.

In this work, AMI was obtained by the reaction of maleic anhydride and β-alanine. Poly(N-alaninylmaleimide) [Poly(AMI)] and poly(N-alaninylmale-imide-co-methacrylic acid) [poly(AMI-co-MA)] were prepared by the photopolymerizations. The structures of monomeric AMI, poly(AMI), and poly(AMI-co-MA) were identified by IR and ^{1}H-NMR spectroscopies.

In vitro cytotoxicities of synthesized polymers were evaluated against mouse mammary carcinoma cell (FM-3A/s), mouse leukemia cell (P-388/s), and human histiocytic lymphoma cell (U-937/s). *In vivo* antitumor activities against sarcoma 180 were also investigated using Balb/C mice bearing tumor.

EXPERIMENT

Materials

β-Alanine and dimethoxybenzoin (DMB) were used as received from Junsei without further purification. Maleic anhydride (MAH), 2-butanone, toluene, and mathacrylic acid (MA) were purified by conventional purification methods. P-388, FM-3A, U-937 cells as target cell lines for *in vitro* test were used. For the *in vivo* test, Baib/C mice and sarcoma 180 cell lines were purchased from the Center of Genetic Engineering (Korea Institute of Science and Technology).

Instruments

IR spectra were taken on a Jasco FT/IR-5300 spectrophotometer using a KBr disc. ^{1}H-NMR spectra were recorded on a FT-300 MHz Bruker A-3000 spectrophotometer. NMR spectra were taken in DMSO-d_6 and TMS was used as

an internal standard. The average molecular weights were measured by a Water-410 gel permeation chromatography (GPC). Elemental analyses were carried out with a Carlo Erba model EA1108 analyzer.

Synthesis of AMI

A solution of MAH (19.6 g, 0.2 mol) in acetic acid (90 mL) was added to a solution of β-alanine (17.8 g, 0.2 mol) in acetic acid (225 mL), and the solution was stirred at room temperature for 3 hrs. The white precipitate was filtered, washed with methanol (100 mL), and dried. The white powder was recrystallized from water to give pure alaninyl maleamic acid (AMA) (Yield; 88%). The melting point of AMA was 161℃. A mixture of 2.6 g (0.015 mol) of AMA and 2.9 g (0.028 mol) of triethylamine in 400 mL of dry toluene was refluxed with concomitant removal of the produced water through a Dean-Stark apparatus for 2 hrs. The toluene solution containing the reaction product was decanted from the brown-colored oil. Toluene was removed by evaporation to give the triethylammonium salt of AMI as a hygroscopic solid. The solid was acidified to pH 2 with HCl, extracted with ethyl acetate, and dried with anhydrous $MgSO_4$. Ethyl acetate was removed *in vacuo* to give AMI (Yield: 30%). The melting point of AMI was 105℃. Elemental Analysis: Cal.(%) for $C_7H_7NO_4$: C, 49.7; H, 4.17; N, 8.28. Found(%) : C, 48.6; H, 4.97; N,7.64.

Syntheses of Polymers

Synthesis of Poly(AMI)

0.85 g of AMI and 0.027 g of DMB as an initiator were dissolved in 18 mL of 2-butanone and acetone (1V/2V) and the solution was introduced into a dry pyrex polymerization tube. After the solution was degassed twice by purging with purified N_2 gas, the tube was sealed and placed in a photochemical chamber reactor using 313 nm lamps at 25 ± 1.0℃ for 72 hrs. The polymer solution obtained was precipitated in diethyl ether. The precipitated polymer was filtered and washed twice with 2-butanone and acetone (1V/2V). Then the polymer was collected by filtration and dried until a constant weight under vacuum (Conversion; 35%).

Synthesis of Poly(AMI-co-MA)

Poly(AMI-co-MA) was prepared by the photocopolymerization of AMI and MA with DMB as an initiator. 0.85 g (5 mmol) of AMI, 0.215 g (2.5 mmol) of MA, and 0.041 g of DMB were dissolved in 18 mL of 2-butanone and acetone (1V/2V) and the solution was introduced into a dry pyrex polymerization tube. After the solution was degassed twice by purging with purified N_2 gas, the tube was sealed and placed in a regulated photochemical chamber reactor using 313 nm lamps at 25±1.0℃ for 72 hrs. The polymer solution obtained was precipitated in diethylether. The precipitate was filtered and washed twice with 2-butanone and acetone (1V/2V). Then the

polymer was collected by filtration and dried until a constant weight under vacuum (Conversion; 27%). The elemental analysis found (%): C, 23.07; H, 2.37; N, 6.85.

Determination of Molecular Weight

Number average molecular weight (Mn) was measured by GPC using a microstyragel column and monodisperse polystyrene as a standard at 40℃. DMF was used as an eluent.

Elemental Analysis of Poly(AMI-co-MA)

The content of AMI moiety in copolymers were calculated from C, N, and H data.

Biological Activity Test

In vitro Cytotoxicity of AMI and its Polymers

The *in vitro* cytotoxicity of AMI and its polymers was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium (MTT) assay.[12] The assay is dependent on the cellular reduction of water-soluble MTT by the mitochondrial dehydrogenase of vial cells to a blue water-nonsoluble formazan crystal product which can be measured spectrophotometrically. Following appropriate incubation of cells (J-82, P-388, FM-3A and U-937 cells) in the presence or absence of synthetic polymers, MTT was added to each well and incubated at 37℃ for additional 4 hrs before processing as described below. For cell growth, serially increasing cell numbers were plated in different columns across 96-well microtiter plates. Well grown cells were harvested, counted and inoculated at the concentrations of 2×10^4 cells/ml into 96-well microtiter plates. After 24 hrs, synthetic polymer were applied to triplicate culture wells and the cultures were incubated at 37℃ for 3 days. The cultured cells were mixed with 20 μl of MTT solution (5 mg/ml in phosphate buffer solution; KCl 0.2 g, KH_2PO_4 0.2 g, NaCl 8.0 g, Na_2HPO_4 1.15 g, $MgCl_2$ 0.101 g/l, pH 7.4) was added to the microculture wells. After 4 hrs of incubation at 37℃, the supernatant was removed from each well and 100 μl of 100% DMSO was added to solubilize the formazan crystals which were formed by the cellular reduction of MTT. After a thorough mixing with a mechanical plate mixer, absorbance spectra was measured on ELISA Processor II Microplate Reader at the wavelength of 570 nm and a reference wavelength of 650 nm (absorbance peak for DMSO). All measurements were carried out in triplicates. There was a good reproducibility between replicate wells with standard errors $<$ +10%.

Antitumor Activity of AMI and Its Polymers

To evaluate the antitumor activity of AMI and its polymers, mice bearing sarcoma 180 tumor cells were used. Balb/C mice were first intraperitonearly implanted with sarcoma 180 cells (2×10^5). The animals were then treated with a saline of sample at days 1-4. Three different dosages were tested: 0.8, 80, and 800 mg/kg. For

comparison, antitumor activities of free 5-fluorouracil (5-FU) also were tested by the same method. A control group was divided into two groups. One group was treated with sarcoma 180 cells along with the same volume of saline and the other group was treated with sarcoma 180 cells. The ratio (T/C) of survival times of the polymer-treated (T) to that of control groups (C) was used as the index of the antitumor acitivity. Each group consisted of 10 animals.

RESULTS AND DISCUSSION

Characterization of Monomer and Polymers

The IR spectrum of AMA showed characteristic absorption peaks at 3500 -2700 (-OH in acid group), 3290 (NH), 1715 (C=O), and 1620 (-CH=CH-). ^{1}H-NMR spectrum of AMA showed methene protons in double bond at 6.42 ppm, methylene protons at 3.37 and 2.52 ppm, and a proton of carboxylic acid at 9.10 ppm.

The IR spectrum of AMI showed characteristic absorption peaks at 3400-3100 (-OH in acid group), 1775 and 1690 (C=O), and 1620 (-CH=CH-). ^{1}H-NMR spectrum of AMI showed methene protons in double bond at 7.01 ppm, methylene protons at 3.61 and 2.50 ppm, and a proton of carboxylic acid at 11.7 ppm.

The IR spectrum of poly(AMI) showed characteristic absorption peaks at 3400-3100 (-OH in acid group) and 1775 and 1690 (C=O). ^{1}H-NMR spectrum of poly(AMI) shows methine protons in polymer backbone at 3.31 ppm, methylene protons at 3.65 and 2.52 ppm, and a proton of carboxylic acid at 11.8 ppm.

The IR spectrum of poly(AMI-co-MA) showed characteristic absorption bands at 3400 (-OH in acid group of AMI and MA), 1745 (C=O of AMI), and 1450 cm^{-1} (-CH_3 of MA). The methine protons, methylene protons, and a proton of carboxylic acid of AMI moiety in poly(AMI-co-MA) were characterized by peaks at 3.31, 3.65 and 2.52, and 12.4 ppm. The peaks at 1.04, 2.02, and 12.4 ppm were assigned to methylene protons, methyl protons, and a proton of carboxylic acid of MA moiety in poly(AMI-co-MA).

Solubility of Polymers

Poly(AMI) and poly(AMI-co-MA) were soluble in water, DMF, and DMSO, but insoluble in common organic solvents such as THF, 2-butanone, chloroform, and toluene.

Average Molecular Weights and Compositions of Polymers

GPC measurements of polymers with polystyrene as the calibration standard showed narrow molecular weight distribution. Mn values of poly(AMI) and poly(AMI-co-MA) were 4,200 and 4,000, respectively. AMI content in poly(AMI-co-MA) was 83%.

In Vitro Cytotoxicity of AMI and Its Polymers

Cytotoxicities of AMI and its polymers were evaluated against FM-3A, P-388, and U-937. Table 1 shows the results of *in vitro* cytotoxicity. All of the synthesized polymers showed less cytotoxicity than AMI.

Table 1. *In Vitro* Cytotoxicity of AMI and Its Polymers Against Tumor Cell Lines

Compound	IC_{50} (μg/ml)		
	FM-3A/s	P-388/s	U-937/s
AMI	25	40	48
Poly(AMI)	72	>100	>100
Poly (AMI-co-MA)	96	>100	>100

IC_{50} : 50% inhibitory concentration

In Vivo Antitumor Activity of AMI and Its polymers

Results of *in vivo* antitumor activity of AMI, poly(AMI), and poly(AMI-co-MA) against sarcoma 180 are listed in Table 2. In this table, the antitumor activity of 5-FU is also shown for comparison.

The life span of mice treated with 5-FU was longer than that of the control group at low doses (34 and 40% increase at dosages of 0.8 and 80 mg/kg), but 5-FU reduced life span by 61% at a high dose (800 mg/kg). 5-FU showed an efficient antitumor activity at low doses but it appeared to show undesirable toxicity at a high dose.

The life span of mice treated with polymers was longer than that of 5-FU and the control group at all doses. At a dosage of 800 mg/kg poly(AMI) and poly(AMI-co -MA) increased life span by 64 and 55%, respectively. At a dosage of 0.8 mg/kg poly(AMI) and poly(AMI-co-MA) increased life span by 74 and 107%, respectively. It means that the antitumor activities of polymers were greater than those of 5-FU and the toxicities of polymers were much less than those of 5-FU.

Table 2. *In vivo* Antitumor Activity of AMI and Its Polymers

Samples	Dose (mg/kg)	Survival times (day)	T/C (%)[a]
Control	-	14.7±2.3	100
	saline	15.7±0.5	100
5-FU	800.0	5.9±0.3	39
	80.0	21.3±1.3	140
	0.8	20.3±1.8	134
AMI	800.0	1.6±1.8	11
	80.0	37.2±3.5	245
	0.8	24.3±2.4	160
Poly(AMI)	800.0	25.0±1.6	164
	80.0	24.0±1.8	158
	0.8	26.5±2.5	174
Poly (AMI-co-MA)	800.0	23.5±1.7	155
	80.0	25.6±1.3	168
	0.8	31.5±1.9	207

[a]T/C(%) = (survival time of treated mice/ survival time of control)×100.

CONCLUSIONS

1. Poly(AMI) was prepared by the photopolymerization of AMI in 2-butanone using DMB as an initiator at 25℃. Poly(AMI-co-MA) was prepared by the photocopolymerization of AMI with MA in 2-butanone using DMB at 25℃.
2. The number average molecular weights of poly(AMI) and poly(AMI-co-MA) were 4,200 and 4,000, respectively.
3. The content of the AMI unit in poly(AMI-co-MA) was 83%.
4. Cytotoxicities of polymers agaist FM-P-388, and U-937 cells were lower than those of AMI.
5. Antitumor activities of samples were

increased in the following order:
5-FU 〈AMI 〈poly(AMI) 〈poly(AMI-co-MA) (at a dosage of 0.8 mg/kg);
5-FU 〈poly(AMI) 〈poly(AMI-co-MA) 〈AMI (at a dosage of 80 mg/kg);
AMI 〈5-FU 〈poly(AMI-co-MA) 〈poly(AMI) (at a dosage of 800 mg/kg).

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